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(71) Applicant *(for all designated States except US)*: **MIRAGENE, INC.** [US/US]; 16632 Milliken Avenue, Irvine, CA 92606 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **O'NEILL, Michael, T.** [GB/US]; 17985 Via Buena Vida, Yorba Linda, CA 92886 (US). **LEBRUN, Stewart, J.** [US/US]; 384 Santa Louisa, Irvine, CA 92606 (US).

(74) Agent: **ALTMAN, Daniel, E.**; Knobbe, Martens, Olson & Bear, LLP, 2040 Main Street, Fourteenth Floor, Irvine, CA 92614 (US).

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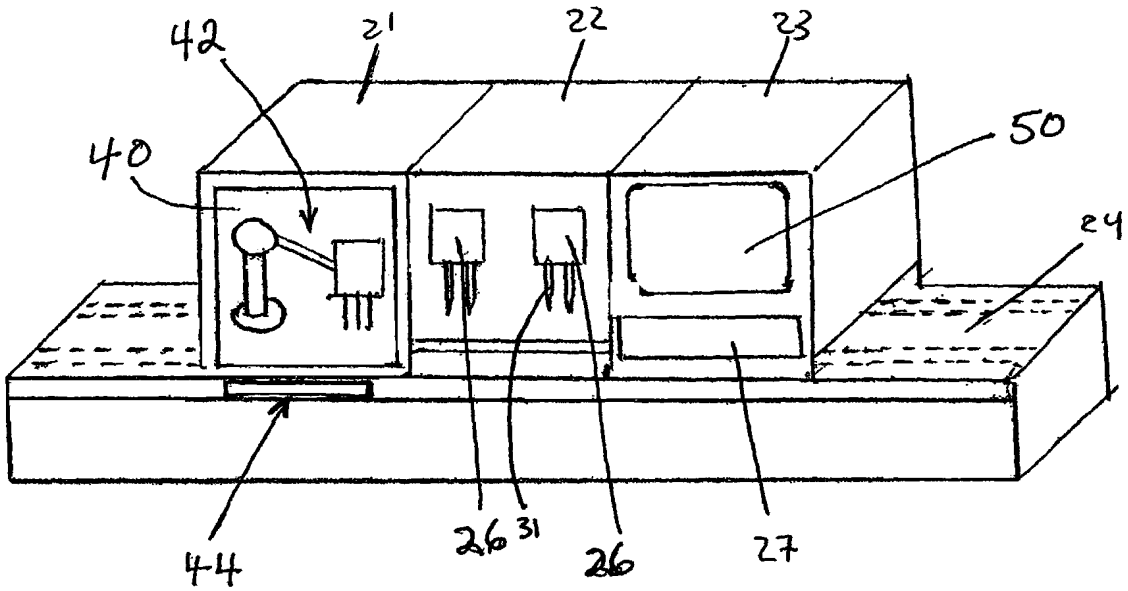
(54) **Title:** INTEGRATED SYSTEM FOR PRINTING, PROCESSING, AND IMAGING PROTEIN MICROARRAYS



(57) **Abstract:** The present invention relates to the assembly and integration of individual components to provide a low-cost system to study biological reactions. The system comprises an optimized substrate surface in a microarray format, a printing station, a processing station and an imaging station, with robotic operations that allow low-cost automated printing, processing, and imaging of protein microarrays.

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INTEGRATED SYSTEM FOR PRINTING, PROCESSING, AND IMAGING PROTEIN MICROARRAYS

Background of the Invention

Field of the Invention

In one aspect, the present invention relates to a system for printing, processing, imaging and analyzing interactions among biomolecular samples. In another aspect, the present invention relates to the integration of individual components to create an automated system for preparing protein microarrays, assaying interactions between the proteins on the array and test substances, and detecting and quantifying the interactions.

Description of the Related Art

It has been said that there is "no PCR for proteins." This illustrates one of the major challenges for protein microarrays. While arrays can hold tens-of-thousands of features, traditional methods make it difficult to purify these individual proteins. Accordingly, it is not surprising that there has been a recent surge in interest in the development of protein microarrays for diagnostic applications (Wilson, D.S. and S. Nock. Recent developments in protein microarray technology. *Angew Chem Int Ed Engl* 2003 Feb3;42(5):494-500; Ng, J.H. and L.L. Ilag. Biomedical applications of protein chips. *J Cell Mol Med* 2002 Jul-Sept;6(3):329-40; Eickhoff, H., Z. Konthur, A. Lueking, H.Lehrach, G.Walter, E. Nordhoff, L. Nyarsik and K. Bussow. Protein array technology: the tool to bridge genomics and proteomics. *Adv Biochem Eng Biotechnol* 2002;77:103-12; Talapatra, A., R. Rouse and G. Hardiman. Protein microarrays: challenges and promises. *Pharmacogenomics* 2002 Jul;3(4):527-36), as protein and antibody microarrays have the potential to serve as valuable tools for drug development and diagnostics (Huels, C., S. Muellner, H.E. Meyer and D.J. Cahill. The impact of protein biochips and microarrays on the drug development process. *Drug Discov Today* 2002 Sept 15;7(18 Suppl):S119-24). Particularly exciting work has been done on printed antibodies and capturing proteins that serve as clinical markers for cancer (Krieg, R.C., C.P. Paweletz, L.A. Liotta and E.F. Petricoin 3rd. Clinical proteomics for cancer biomarker discovery and therapeutic targeting. *Technol Cancer Res Treat* 2002 Aug;1(4):263-72). In fact, entire complex tissue arrays have been used to identify disease markers for prostate cancer (Zellweger, T., C. Ninck, M. Mirlacher, M. Annefeld, A.G. Glass, T.C. Gasser, M.J. Mihatsch, E.P. Gelmann and L. Bubendorf. Tissue

microarray analysis reveals prognostic significance of syndecan-1 expression in prostate cancer. *Prostate* 2003 Apr 1;55(1):20-9), renal duct and regulatory proteins (Legato, J., M.A. Knepper, R.A. Star and R. Mejia. Database for renal collecting duct regulatory and transporter proteins. *Physiol Genomics* 2003 Apr 16;13(2):179-81), and proteins of the normal placenta (Batorfi, J., B. Ye, S.C. Mok, I. Cseh, R.S. Berkowitz and V. Fulop. Protein profiling of complete mole and normal placenta using ProteinChip analysis on laser capture microdissected cells. *Gynecol Oncol* 2003 Mar;88(3):424-8). Limited work has been done on assays involving enzymatic activity in the two-dimensional microarray format (Chen, G.Y., M. Uttamchandani, Q. Zhu, G. Wang and S.Q. Yao. Developing a Strategy for Activity-Based Detection of Enzymes a Protein Microarray. *Chembiochem* 2003 Apr 4;4(4):336-339).

Current methods to print biological samples onto a microarray format include manual printing and robotic printing, however, the robotic printing devices, while semi-automated, are generally medium to high cost. For example, although an instrument from Gene Machines is capable of printing up to 50 slides in several hours, the cost of the device is often prohibitive to the individual researcher.

Current methods of scanning microarrays include chemiluminescence and fluorescent scanning devices. In addition to being relatively expensive, these devices are generally only capable of scanning one microarray slide at a time, thus causing a bottleneck between the printing and scanning steps.

Because of the large capital investment in equipment to do both printing and scanning, the individual researcher generally must rely on the institution's core facility, which may slow down research activity. In addition, there is often a high overhead charge, which may have to come out of his or her operating grant.

In another context, many of the substrates currently available for microarray slides, while suitable for DNA and RNA samples, are not optimized for protein applications.

A related and additional concern is the proper identification and tracking of each individual microarray slide, and this concern is greater when the slides are transported from one department to another and back again.

Accordingly, there remains a long-felt and unanswered need for an affordable, integrated high throughput system for automatically printing, processing, and imaging protein microarrays.

Summary of the Invention

In accordance with a preferred embodiment of the present invention, a system is disclosed for printing, processing and imaging biomolecular interactions. The system comprises: (1) a template comprising at least one well configured to hold a microarray substrate; (2) a printing station comprising an automated microarray printer in a climate-controlled compartment for applying a plurality of biomolecular markers in an addressable manner to the microarray substrate; (3) a processing station comprising an automated liquid dispensing and mixing robot for adding, mixing and/or extracting liquid reagents from the microarray substrate, wherein the liquid reagents comprise at least one test sample and a developing reagent, and wherein the developing reagent is capable of generating a detectable signal when the test sample binds to a biomolecular marker on the microarray substrate; and (4) an imaging station comprising a flatbed digital scanner for digitizing the detectable signal(s), such that a digitized image of the microarray substrate is formed, wherein the imaging station has a microprocessor comprising imaging software adapted to quantify and analyze the digitized image.

In one preferred variation, the above-described system further comprises a mechanized conveyor system adapted to transport the template from the printing station to the processing station to the imaging station. Preferably, the system also comprises control software with a user interface. The control software is adapted to provide the user with hands-free control of printing, processing, imaging and transport parameters.

In one embodiment, the climate-controlled compartment regulates at least one of temperature, humidity, and gas content. Preferably, the temperature in the climate-controlled compartment is maintained between about 0° C and about 37° C. More preferably, the temperature is maintained between about 4° C and about 8° C.

In on preferred variation, the automated microarray printer utilizes a printing buffer in applying the biomolecular markers to the microarray substrate. Preferably, the printing buffer comprises KPO_4, NaCl, methanol, and DTT.

In one preferred aspect of the present invention, the microarray substrate is mounted on a column within each well. In a variation, each well further comprises a drain configured to facilitate removal of liquid reagents from the microarray substrate during processing.

In a preferred embodiment of the present invention, the microarray substrate further comprises a PVDF membrane.

In one preferred aspect of the present invention, the processing station further comprises a vacuum source adapted to facilitate vacuum-assisted drainage and vacuum drying of the microarray substrate. In another preferred aspect, the processing station is further adapted to position a protective cover over the template. Preferably, the protective cover is adapted to seal the wells. The protective cover may also comprise sealable ports adapted to allow a tubular member from the automated liquid dispensing and mixing robot to enter the well in order to add, mix and/or extract liquid reagents from the microarray substrate.

In one preferred embodiment of the present invention, the imaging station further comprises a cooling device.

In another preferred embodiment of the present invention, the biomolecular markers are proteins or nucleic acids. The biomolecular markers are preferably antibodies, antigens or allergens.

In one aspect of the present system, the template preferably comprises from 1-96 wells. In another aspect, the developing reagent comprises an enzyme-linked antibody adapted to generate a colorometric change upon addition of a substrate.

A smaller system is disclosed in accordance with another preferred embodiment of the present invention. This system is for processing and imaging biomolecular interactions, and comprises: (1) a template comprising at least one well configured to hold a pre-printed microarray substrate, wherein the microarray substrate comprising a plurality of biomolecular markers immobilized in an addressable manner on a PVDF membrane; (2) a processing station comprising an automated liquid dispensing and mixing robot for adding, mixing and/or extracting liquid reagents from the microarray substrate, wherein the liquid reagents comprise at least one test sample and a developing reagent, and wherein the developing reagent is capable of generating a detectable signal when the at least one test sample binds to one of the plurality of biomolecular markers on the microarray substrate; and (3) an imaging station comprising a flatbed digital scanner for digitizing the detectable signal(s) such that a digitized image of the microarray substrate is formed, and a microprocessor comprising imaging software adapted to quantify and analyze the digitized image.

Preferably, the smaller system also has a mechanized conveyor system adapted to transport the template from the processing station to the imaging station. In a preferred variation, the smaller system also has control software with a user interface, wherein the control software is adapted to provide the user with hands-free control of processing, imaging and transport parameters.

Brief Description of Drawings

Figure 1 depicts an integrated system for printing, processing and detecting protein interactions in a high throughput microarray format.

Figure 2 depicts a smaller integrated system for automatically processing and detecting protein interactions on pre-assembled microarrays.

Figure 3 is a top view of a prefabricated multiwell microarray template in accordance with one preferred embodiment of the present invention.

Figure 4 is a side view of the prefabricated multiwell microarray template of Figure 3 showing a row of wells having a raised column bearing the microarray substrate.

Figure 5 is a side view of a single well of the microarray template, showing liquid delivery, mixing and extraction needles disposed within the well and surrounding the raised column.

Figure 6 is a protein microarray image showing spot quality and consistency generated by printing protein antigens using a TeleChem SPOTBOT™ printer on a Miragene Z-GRIP™ PVDF substrate, developed using an enzyme-linked colormetric reaction, and digitized using a flatbed scanner.

Figure 7 is a magnified view of a protein microarray image showing a dose-responsive increase in protein spotting generated using a BioDot non-contact printer on Miragene Z-GRIP™ PVDF substrate, developed using an enzyme-linked colormetric reaction, and digitized using a flatbed scanner.

Figure 8 is a top view of a protective cover for the microarray template shown in Figure 3, showing four needle holes adapted to allow the liquid delivery, mixing and extraction needles to access individual wells without disturbing the substrate-bound reactants which are positioned within the centers of the wells on raised columns.

Figure 9 is a side view showing robotic delivery and extraction of materials for a single well in the multiwell template.

Figure 10 shows a scanned microarray image generated using a TeleChem SPOTBOT™ printer and a Miragene Z-GRIP™ substrate.

Figure 11 shows the use of gridding to isolate individual spots.

Figure 12 shows an expanded view of the interaction among the multiwell template, the protective cover and the robotic reagent handling device.

Detailed Description of the Preferred Embodiment

In one embodiment, the present invention relates to the assembly and integration of individual components to provide a low-cost system to study biological interactions. The system comprises optimized substrate surfaces in a microarray format that allows for low-cost printing, processing, detection and tracking of biological samples within the same system. The system preferably comprises a robotic microarray printer located in a climate-controlled compartment, a robotic sample and reagent handling compartment for processing the microarrays, an automated scanning and detection system, and an automated template transportation mechanism.

In a basic embodiment of the present invention, an integrated system is disclosed which comprises: (1) an automated microarray processing compartment, comprising a robotic liquid handling device for performing repetitive processing operations (e.g., blocking, washing, addition of test samples, addition of developing reagents, etc.); in operable linkage with (2) an automated imaging compartment, comprising a conventional flatbed scanner for digitizing the spots on the developed microarray slide, and the software for analyzing the image. These components are adapted to accommodate at least a single microarray slide.

In preferred embodiments of the integrated system, the printing component can print from about 1 and 14 microarray substrates (or slides) in one run, and more preferably, about 1 to 32, and most preferably, about 1 to 96. To facilitate high throughput, addressable printing, the system preferably utilizes a multiwell template adapted to securely hold in a fixed position a plurality of microarray substrates.

Similarly, both the processing and imaging stations are preferably configured to process and scan, respectively, from about 1-14 microarray substrates, more preferably, from about 1-32, and most preferably, from about 1-96 microarray substrates per run. Again, to facilitate the high throughput, addressable processing and imaging, the processing

and imaging stations are preferably configured to accommodate the multiwell template, which provides accurate and consistent array positions.

In a preferred aspect of the present invention, the individual microarray substrates can incorporate a barcode identification number for ease of tracking.

In another aspect of the present invention, immunochemistry protocols for optimized detection of the various biological reactions are disclosed.

Described herein is a system of reagents, hardware and software that in combination allow protein, DNA or RNA samples to be spotted, processed and detected in a high throughput microarray format. Preferably, the hardware components are integrated into a single system that includes: a conveyor mechanism for moving the microarray slides (or multiwell template) from the printing station to the processing station to the imaging station, robotic handling devices, reagent bottles, vacuum pump, mixing devices, fluidics delivery pump, cooling and humidification system, microarray scanner and enclosure with adequate insulation, vibration reduction and access ports. These components make up the "core hardware," as described in detail below. In addition to the appropriate hardware, microarray templates (or trays), reagents and an integrated computer with appropriate software and use protocols are disclosed.

Using commercially available or custom designed robotic handling devices, an integrated system provides hands-free operation to print, process, detect, scan and analyze biomolecular interactions. Such a system reduces the problems of laboratory handling and contamination, increases efficiency, and lowers operational costs.

The system preferably comprises a prefabricated microarray template (tray or cartridge) containing multiple wells. For example, a microarray tray or cartridge may contain at least 96 wells. Each well preferably contains a membrane attached to a rigid surface in the middle of the well, and an optional drain at the bottom of each well. In a preferred embodiment, the microarray well minimizes sample and reagent volume requirements by at least one order of magnitude.

The microarray template is preferably loaded onto the system and transported to the first station for printing of the features of interest. Such features include, but are not limited to, DNA, RNA, and proteins such as antigens, antibodies, and allergens. Such features are loaded onto the membrane surfaces of the individual microarray substrates. For printing proteins, the temperature is preferably maintained between about 0° C and 37° C, more

preferably, between about 4° C and 24° C, and most preferably, between about 4° C and 8° C.

In one preferred embodiment, a robot can print features onto the membranes. For example, such printing can be achieved by contact printing, non-contact printing, piezoelectric printing or other similar printing methods known by those skilled in the art. Available robotic printers include TeleChem's SPOTBOT™ contact printer and BioDot's non-contact automated printer.

After printing, the microarray template is preferably transported to the next compartment via a conveyor belt. In one preferred embodiment, microarrays are allowed to air-dry either in the climate-controlled printing compartment, or between the printing and processing stations, prior to sample processing.

In one preferred embodiment, the printed arrays are prepared for processing by covering the microarray template with a sealed cover. The placement of the protective cover on the microarray template is preferably automated. In a particularly preferred embodiment, the protective cover comprises access ports to allow tubular members from the processing robot (e.g., syringe needles, metal or plastic cannulas, etc.) to access the wells within the template.

Using the robotic dispensing, mixing and extraction devices, various materials may be preferably added to the samples. For example, reagents and washing and blocking buffers can be added to, mixed with, and removed from the features of interest which are immobilized on the membrane of the microarray substrate.

In one embodiment, after completing the various blocking and washing cycles, a test sample is added and then removed after a desired interval. Subsequently, a developer is added and then removed after a desired interval. After the developer is removed, air-drying preferably takes place.

The microarrays are preferably transported to the next compartment where scanning and detection of the interactions are studied using the imaging software. In one preferred embodiment, the results may be presented on a screen and/or printout, or saved on a disk.

Core Hardware

Figure 1 shows a diagram of one possible core hardware configuration with the individual components integrated into a complete system. As illustrated, the core hardware preferably contains three compartments coupled together and mounted around a conveyor

system **24** to transport the microarray slides (or multiwell templates) from compartment to compartment.

A conveyor system in accordance with a preferred aspct of the core hardward can be any commercially available or custom designed transport system. For example, an automated conveyor system is disclosed in US Pat. No. 5,736,101, which is incorporated herein in its entirety by reference thereto.

The multiwell microarray template is preferably loaded onto a conveyor mechanism **24**, e.g., rails, and fed into the printing compartment **21** of the integrated system. The microarray substrate is indexed into position for printing. The printing station **21** preferably contains a climate-controlled compartment **40** for temperature and humidity control during microarray printing. Printing is preferably accomplished using an automated printer **42** which apply the biomolecular markers (also called features, which can comprise any molecules, preferably, capture molecules having recognition epitopes of interest) via conventional contact printing, non-contact printing (spraying), piezoelectric printing or other similar printing methods known by those skilled in the art. In one embodiment, a cooling device (e.g. peltier) **44** is installed directly below the microarray template to control the temperature after the microarray template has been loaded into the first station.

In a particularly preferred embodiment, environmental controls allow chemistries at wide ranges of temperatures and humidities. For example, temperatures may be selected between about 0° C and 37° C, more preferably 4° C and 24° C, and most preferably between about 4° C and 8° C. The cooling device may be selected from conventional refrigeration devices which are adapted to cool the entire compartment or platform coolers, such as a peltier **44**, which are adapted to cool the microarray templates. A conventional humidifier may be used to maintain moisture within a range of about 10% to 100% humidity, more preferably about 50% to 100% humidity, and most preferably about 70% to 100% humidity.

In addition to temperature and humidity control, other preferred embodiments may incorporate additional environment controls. Such additional controls may include, but are not limited to: zero oxygen to prevent oxidation of samples, regulated carbon dioxide to regulate pH, and the addition of inert gas such as nitrogen or noble gas to reduce sample degradation and microbial activity.

Additional components such as buffer and sample vials, or multiwell (e.g., 96 or 384 well) dishes, that hold materials are preferably located within the temperature and humidity controlled compartment. The wells may contain, for example, buffers for washing the printing pins/heads, or the biomolecular markers or capture molecules (e.g., antibodies, antigens, allergens, etc.).

Examples of commercially available automated printers include TeleChem's SPOTBOT™ contact printer and BioDot's non-contact, spray printer.

The processing station **22** preferably contains one or more robotic sample and reagent handling devices **26** for dispensing, mixing, and extracting materials in and out of the microarray template. Typically, such liquid handling robotic systems utilize hollow cannulas, e.g., syringe needles **31**, for adding and aspirating liquid reagents from the individual wells in the template. In one particularly preferred embodiment, this station houses the mechanism that places a protective cover over the microarray template. In preferred embodiments, where a protective cover is used to seal the microarray substrates within their wells in the template, compartment climate-control means may be optional.

Examples of automated liquid processing devices are disclosed in US Pat Nos. 6,044,876, 6,367,669, 6,569,385 and 5,736,101, which are all incorporated herein in their entirety by reference thereto.

The imaging station **23** preferably contains at least the scanner **27**, microprocessor, electronic storage capability, printing and/or display, and software (e.g., an integrated computer system **50**). In one preferred embodiment, the scanner **27** is installed above the conveyor mechanism, with the scanning surface facing downward, toward the microarrays.

In another preferred embodiment illustrated in **Figure 2**, the integrated system contains only the processing **22** and imaging **23** stations, as well as the conveyor mechanism **24** for automated hand-free operation. This smaller, less costly, system may be desired by users who purchase or otherwise obtain pre-printed microarrays (e.g., commercially available and/or custom prepared microarrays).

Preferred System Variations

In one preferred embodiment, a prefabricated microarray template, tray, or cartridge, (**Figures 3 and 4**) is designed to increase the number of individual microarray surfaces from a single slide format (as disclosed in co-pending US Pat Appn. No. 10/376,351; incorporated herein in its entirety by reference thereto) to a multi-microarray format using a

hydrophobic membrane substrate, comprising a plurality of wells **28**, each adapted to hold a microarray substrate **29** in a fixed position within the well. The illustrated template contains 96 individual wells **28**. As can be seen from the side views (**Figures 4 and 5**), the microarray substrates **29** are suspended on a raised columns **30** above the floor of the well. As shown in **Figure 5**, this well design facilitates the insertion of liquid dispensing, mixing and aspirating needles/pins **31** into the wells without disturbing the biomolecular reactions occurring on the surface of the microarray substrates **29** raised on the columns **30**. The microarray substrates preferably comprise polyvinylidene difluoride (PVDF) membranes. For example, the substrate surface preferably falls between 0.10 cm^2 to 2.00 cm^2 in surface area allowing the printing of approximately 225 to 4500 features; (**Figure 5**).

Printing heads such as the TeleChem contact printing stealth pins or BioDot non-contact printing heads have been used in accordance with variations of the present invention. Examples of spot consistency on Z-GRIP™ PVDF microarray substrate (Miragene, Inc.) is illustrated for both contact printing pins (**Figure 6**) and non-contact spray printing (**Figure 7**; increasing spot volumes are shown).

With reference to **Figure 8**, an embodiment of the protective cover **52** is illustrated with 4 access ports **32** positioned so as to allow access of liquid delivery needles/pins **31** to the wells **28**, while straddling the microarray substrate **29** fixed on top of the column **30** (see **Figures 3-5**). In a preferred embodiment, the protective cover **52** is further adapted to seal the individual wells **28**. The wells may be configured to have a drain which can be actuated by turning on a vacuum source to facilitate aspiration of washes, etc. The vacuum source can also be applied to the wells, when the protective cover effectively seals the well—thereby facilitating vacuum drying. Alternatively, the needles/pins can aspirate the liquid washes, etc. to be discarded.

In one preferred embodiment, sample processing typically involve the following steps. The microarray wells are filled with about 0.1 to 1.0 ml, preferably about 0.5 ml of a blocker solution and agitated by sonification, vibration or gentle shaking. With reference to **Figure 9**, there is shown a side view of the template wherein a robot is illustrated delivering into a well **28** via needles **31** a reagent from a vial **33**. Unknown sample or control (preferably about 1 µl of undiluted sample), such as a patient serum sample, primary antibody, etc., is added to blocker solution and agitated. After completing the mixing cycle the solution is discarded by needle aspiration/extraction or draining, as described above.

The substrates are then preferably washed and mixed several times with wash buffer (e.g., about 0.5 ml), and the wash solutions are discarded. Appropriately diluted buffer containing a developing reagent(s) (e.g., about 1 µl undiluted) is added to each well and agitated. The developing solution is then discarded. Developing reagents may be secondary antibodies, preferably linked to an enzyme which can catalyze a reaction that results in the development of a detectable (preferably, colorometric) change upon addition of the enzyme substrate. Additional washes may be desired to minimize background. Each well may be flushed with water for approximately 2 minutes to stop any further color development. The substrates are preferably then air-dried and the microarray template is transported to the imaging station.

With reference to **Figure 12**, there is shown an expanded view of the template **10**, the protective cover **52** and the robotic liquid dispensing, mixing and aspirating device **26**. It can be seen that the needles **31** enter the wells **28**, via the access ports **32**, straddling the column **30** with the microarray substrate **29** affixed thereon. Reagent can then be delivered from the vial **33** into the wells.

In another embodiment the system consists of only two compartments (**Figure 2**), without the first printing compartment, for use with pre-printed microarrays to be typically used in diagnostic applications. Custom made microarray templates/trays with specific panels of disease related biomolecular markers may be supplied for diagnostic tests such as autoimmune disease, infectious disease and allergy panels. The microarray template/tray is loaded directly into the sample preparation compartment and processed as described above; after processing the microarray template/tray is transported into the imaging station for detection and analysis of results.

Microarrays in accordance with preferred aspects of the present invention

Most traditional protein studies have involved wet chemistries and porous membranes such as the polymer polyvinylidene difluoride (PVDF), which is widely used in techniques such as western blot. Experiments were carried out to evaluate if proteins could be immobilized on the surface of a membrane such as PVDF when it is in a dry state.

PVDF was adhered to a glass support using an inert double-sided adhesive microfilm. Proteins (more specifically antigens) were spotted onto the dry surface of the PVDF and after drying were allowed to interact with other proteins (more specifically antibodies) with a conjugated secondary antibody. Results obtained and presented herein

demonstrate that the laminated substrate has proven to overcome all of the aforementioned problems encountered with existing substrates. In addition, the opaque nature of the membrane together with the chemical detection system allows the interactions to be detected and analyzed on a low-cost flatbed scanner using light in the visual wavelength spectrum.

In one embodiment, the present invention provides a protein microarray substrate that can be used in a dry state to immobilize proteins. A hydrophobic membrane is included that immobilizes proteins in a reduced surface area with minimal diffusion across the membrane. The laminated membrane adheres to a glass surface with a double-sided inert adhesive microfilm.

In one embodiment, the present invention can be used with multiple conjugated secondary antibodies such as Alkaline Phosphatase (AP), Biotin Protein A, or enzyme labels such as HRP or fluorescent dyes etc. In one preferred embodiment, the present invention optionally includes a barcode for test and/or sample identification and data archiving.

In one embodiment, the present invention provides a protein microarray with very little background noise. More specifically, the background noise for the Z-GRIP™ PVDF-coated glass slide using the alkaline phosphatase (AP) reaction for detection of proteins, visualized using a conventional flatbed scanner, is less than about 100 lumens. More preferably, the background on the Z-GRIP™ developed as above is between about 50 and 0 lumens. Most preferably, the background is from about 15 to 0 lumens. Similarly little to no background is seen when a fluorescent dye is used for protein detection on the Z-GRIP™ PVDF-coated glass slide and imaged using a fluorescent scanner. In contrast, typical backgrounds seen using commercial protein substrates, e.g., slides with epoxy surface chemistries, are above 200 lumens and usually in the 300 to 400 lumen range.

Maximum signal intensities for the Z-GRIP™ PVDF-coated glass slide in accordance with a preferred embodiment of the present invention using the Alkaline phosphatase reaction for detecting proteins and a conventional flatbed scanner for quantifying spot densities (otherwise referred to herein as "protein imaging"), analyzed using commercial imaging software (e.g., Adobe PHOTOSHOP®) are about 15,000 to 25,000 lumens. Maximum signal intensities for the Z-GRIP™ substrate using fluorescent detection chemistries and a fluorescent scanner are usually about 25,000 lumens. Although

epoxy substrates also produce maximum signal intensities of about 25,000 lumens with either AP or fluorescent detection, because of the relatively high background levels seen with epoxy slides, the Z-GRIP™ PVDF-coated slides have approximately 10-fold greater total dynamic range and signal-to-noise ratios than other protein substrates. Moreover, in accordance with a preferred embodiment of the present invention, background for any detection chemistry on a PVDF-coated rigid support is less than about 1% of the maximal signal intensity, and more preferably, in the range of about 0.1% to about 1%, and most preferrably about 0.1% (e.g., 25 lumens background / 25,000 lumens max signal).

In addition to the advantages discussed above with regard to the higher signal-to-noise ratio seen with a preferred embodiment of the present invention, the Z-GRIP™ PVDF-coated rigid supports also generate enhanced assay sensitivity because the hydrophobic PVDF surface facilitates superior protein spotting/density than the hydrophilic surface chemistries typically used for protein arrays (See e.g., Salinaro *et al.* WO 01/61042 which teaches the criticality of using a hydrophilic surface for biomolecular arrays). As a result of the hydrophobic nature of PVDF, protein samples spotted onto the PVDF surface tend to stay in high density, very discrete micro-spots, which do not spread and diffuse through the polymeric substrate. Thus, the protein density is relatively high compared to proteins spotted onto hydrophilic substrates. As a result of the high density, the concentration of protein does not become limiting on the subsequent detection reactions (e.g., labeled secondary antibody binding). Where protein spots have spread in hydrophilic substrates, the relative protein concentrations are much lower and become limiting on the detection reactions. Consequently, the sensitivity seen using the Z-GRIP™ hydrophobic surface chemistry was observed to be approximately 1000-fold greater than sensitivities obtained with the same proteins and detection reactions on a hydrophilic surface.

In one embodiment, the present invention provides a protein microarray with the capacity to immobilize up to 20,000 proteins in the open array format.

The term "immobilize," and its derivatives, as used herein refers to the attachment of a bioactive species directly to a support member or to a support member through at least one intermediate component. As used herein, the term "attach" and its derivatives refer to adsorption, such as, physisorption or chemisorption, ligand/receptor interaction, covalent bonding, hydrogen bonding, or ionic bonding of a polymeric substance or a bioactive species to a support member. Although the substrate chemistries of the present invention

are adapted to immobilize any proteins, peptides, or polypeptides, in some embodiments of the invention, protein antigens are disclosed as being immobilized. Accordingly, the terms "antigens" and "proteins" are used interchangeably throughout the disclosure unless explicitly otherwise indicated.

Related methods of immobilizing bioactive molecules, in particular, nucleic acids, on polymeric substrates are disclosed in U.S. Patent Nos. 5,897,955 to Drumheller and 6,037,124 to Matson; the disclosures of which are incorporated herein in their entirety by reference thereto.

This work resulted from our attempts to perform immunochemistry using antigens printed by a commercial DNA/RNA/Protein printer. The present inventors found that commercially available substrates and chemistries developed for nucleotides are not optimal for protein binding or immunochemistries. Various derivitized slides including aldehyde, epoxide, amine, L-lysine were not adequate for our requirements. Our suspicion is that binding chemistries utilized to linearize nucleotides for hybridization are not optimal for protein-protein or protein-antibody interactions. It is likely that aggressive binding of these substrates destroys secondary and tertiary protein structures and to the extent these structures are altered, epitopes vital for immuno or protein-protein assays are altered.

PVDF membrane is often used for the western blotting technique. This method involves a pre-soaking step of membrane in methanol to solubilize and the addition of methanol to buffers. The membrane must be kept in the methanol buffer or proteins will not transfer to membrane. This is often the case when there are large areas on a membrane where there was no transfer due to a bubble. In addition to being hydrophobic, PVDF membrane is hard to handle and will not lie flat during printing. These physical and chemical limitations make PVDF membrane an inappropriate and poorly suited surface for protein arrays.

We have developed a method to utilize PVDF membrane, sheets or pellets for immunochemistry and protein-protein interaction studies. Two modifications include: (1) adhering PVDF to a rigid support using an inert double-sided adhesive film or silicone, epoxy or other glues, double sided tape or direct chemical bonding to silanated slides, and (2) a printing buffer that both protects protein three-dimensional integrity and allows adherence to PVDF under dry printing conditions without membrane soaking in solvent (e.g., methanol) and associated diffusion.

One application is the separation of replicate arrays from each other on the same slide to allow patient comparisons or titrations. One or more steps can be performed in the small well then washing and other steps can be performed with larger volumes of solution across the whole slide.

In one embodiment, the present invention provides a three dimensional porous membrane attached to a solid support such as glass with an inert polymer. The three dimensional substrate captures and protects proteins in the porous membrane. The porous membrane has a thickness of greater than about 100 μm, more preferably approximately 100-500 μm, and most preferably between about 100-250 μm. The pore size is any pore size conventionally used for biological materials, particularly peptides and polypeptides. The high-end cutoff pore size is preferably between about 0.1 and 1 μm. More preferably, a pore size of 0.2 or 0.45 μm is used and most preferably a pore size of about 0.45 μm. Note that these pore sizes refer to maximum pore size and that there may be a range of smaller pores, below the cutoff value, present on the membrane. These characteristics help maintain the morphology of the proteins. Proteins spotted onto the substrate surface maintain their integrity, providing increased sensitivity and assay consistency.

In one embodiment, the present invention is an effective tool for studying protein-antibody, antibody-protein, protein-protein and protein-drug interactions.

In one embodiment, the array substrate (Z-GRIP™) is assembled by hand on the laboratory bench. Under clean conditions the protective coating on one side of an inert double-side adhesive film is removed and attached to a solid support such as a glass slide. A sheet of PVDF is placed on the laboratory bench face down with the protective cover still in place. The remaining protective cover on the adhesive film is removed and the solid support is then pressed firmly onto the sheet of PVDF and allowed to dry. Using a sharp instrument, e.g., a razor blade, exacto knife etc., the PVDF membrane is trimmed to the size of the solid support. As an alternative to an inert double-sided adhesive film, other adhesive materials such as silicone, glue or double-sided tape can be used.

In a preferred embodiment the Z-GRIP™ protein microarray substrate is manufactured automatically under clean conditions. A large roll (approximately 1100 inches in length and 11 inches wide) of PVDF (obtained from Millipore Corporation) mounted on a 3.25-inch core is attached to a cutting and lamination machine. The machine automatically laminates a protective film to the upper side of the PVDF and an inert

double-sided adhesive film with extended liner to the backside and cuts the sheets into the preferred size for automatic placement on 3" x 1" glass slides. In alternative embodiments, much smaller microarray substrate slides are affixed to raised columns within multiwell templates (as illustrated in **Figures 3 and 4**).

In another embodiment of the present invention, a layer of PVDF may be formed on a solid support by melting the polymer and applying it to the solid support. Modification of the PVDF chemistry is also deemed to fall within the scope of the present invention. Modifications may include carboxylation, amidization, and introduction of other reactive groups to the PVDF in order to promote immobilization of different bioactive species. In one embodiment, solid PVDF supports may be prepared by molding of the melted polymer.

We have developed a method to utilize PVDF membrane, sheets or pellets for immunochemistry and protein-protein interaction studies. Two modifications include: (1) adhering PVDF to solid support, and (2) administering a printing buffer that both protects protein three-dimensional integrity and allows adherence to PVDF under dry printing conditions, without membrane soaking in methanol. For example, materials used as a protein-immobilizing polymer include, but are not limited to: commercially available PVDF sheets or membranes. PVDF pellets may also be used in some modes of the invention. Solid support materials include, but are not limited to: glass slides, plastic or other flat surfaced material. Adhesive materials used bond the membrane to the solid support include, but are not limited to: commercially available silicon sealant, epoxy or other glue or suitable double sided tape.

In another embodiment of the present invention, a membrane layer of PVDF may be formed on a solid support by melting the polymer and applying and it to the solid support. Modification of the PVDF chemistry is also deemed to fall within the scope of the present invention. Modifications may include carboxylation, amidization, and introduction of other reactive groups to the PVDF in order to promote immobilization of different bioactive species. In one other embodiment, solid PVDF supports may be prepared by molding of the melted polymer.

Microarray studies traditionally used derivitized glass slides with functional groups, such as poly-lysine, amine, and epoxide. One report suggests that ~125-pg of human immunoglobulin is the detection cutoff on an optimized glass substrate. This has low sensitivity compared to Z-GRIP™, which can detect down to 2.5-pg of human

immunoglobulin. Glass substrates give consistent spot deposition, however, immunoreactivity has been found to be variable and less sensitive. Microarray substrates were originally developed for nucleotides, and so have been developed to aggressively bind nucleotides, such that they are linearized and available for hybridization. It is, therefore, surmised that aggressive covalent and ionic interactions between these substrates and proteins result in the deformation of epitopes, and may affect protein assays that are dependent on structure. Another issue with derivitized glass slides is its planarity. This reduces the amount of protein that can be bound to the substrate when compared to the amount that can be bound to a three-dimensional substrate. The amount of protein bound to the substrate directly determines signal intensity and signal to noise ratio.

There has been a movement towards three-dimensional and hydrophilic substrates for protein microarrays. These are efforts to both increase the surface area and maintain the three-dimensional structure of the proteins. However, it is not clear that hydrophilic substrates are acceptable for contact printing, as pins load by wick action. It is hypothesized that when protein solutions contact a hydrophilic substrate, the initial contacts result in a larger transfer of protein sample. This can result in uneven spot deposition, making quantification quite difficult.

Bonding of membrane substrate to slide or multiwell template

In one embodiment the mini Z-GRIP™ protein array may be assembled by hand on the laboratory bench. Under clean conditions a sheet of PVDF is preferably placed on the laboratory bench face up with the protective cover still in place then a single-hole punch is pressed into the PVDF to form a sub-array disc. A double-sided adhesive polymer is preferably placed on the laboratory bench and the protective film is removed from the top-side. The hole punch with the disc is then preferably pressed firmly onto exposed adhesive polymer. The remaining protective film is preferably removed from the double-sided adhesive polymer and the disc with adhesive is pressed onto the column in the middle of the well.

In a preferred embodiment, the PVDF disc **29** is installed on the well column **30**. The protective cover, which may be used to protect the substrate during shipping and storage is removed from the template, exposing the PVDF disc, which is ready for printing. As an alternative to an inert double-sided adhesive film, silicone, glue or double-sided tape can be used.

In a preferred embodiment the mini Z-GRIP™ protein array is manufactured automatically under clean conditions. A roll of PVDF, mounted on a core, is preferably attached to a cutting and lamination machine. The machine automatically laminates a protective film to the upper side of the PVDF, and cuts the sheets into the preferred size for automatic placement of the PVDF discs onto the rigid well columns in the microarray tray.

The mini Z-GRIP™ microarray preferably provides a three dimensional porous membrane **29** attached to a well column **30** with an inert polymer. The three dimensional substrate preferably captures and protects proteins in the porous membrane.

In one preferred embodiment, the porous membrane has a thickness of approximately 300-500μm with a pore size of approximately 0.040 – 0.050μm, and these characteristics preferably help maintain the morphology of the proteins.

In another preferred embodiment, a drainage mechanism is included in each well. In one preferred embodiment, the drainage mechanism comprises a hole located within each well. In another, the hole has a vacuum actuated one-way cover or flap, which seals the bottom of the well under ambient pressure, but which allows drainage under vacuum.

Printing

In one particularly preferred embodiment, a printing buffer optimizes printing consistency and immunochemistry. The printing buffer preferably includes, but is not limited to: KPO_4 buffer (maintain desired pH), NaCl, Methanol and DTT. For example, a particularly preferred embodiment of the buffer includes, but is not limited to: 20 mM KPO_4 (pH 7.4), 100 mM NaCl, 10% Methanol, and 0.01 % DTT. In another preferred embodiment, the buffer includes 20% methanol. After printing is complete, arrays can be used for protein-protein interaction studies or immunochemistry applications.

The test system described herein utilized the Telechem Stealth Microspotting Pins, or SMP3 (specifically that with a 75-μm tip), in conjunction with the Telechem Stealth Printhead, or SPH48, to print protein microarrays. This system was originally developed to print nucleotides, so the application to proteins may be a cause for concern, especially with regards to pin clogging and washing protocols. When printing, the Stealth pin goes through a wash/dry cycle, and in some cases, a sonication cycle prior to loading the protein (in buffer) solution. Loading takes only a few seconds, which is enough time for the entire slit pin chamber to fill with protein solution. The protein solution is then robotically contact-printed onto glass preprint slides. This allows any inconsistency of droplet formation at the

pin tip to be removed, resulting in a consistent meniscus formation at the pin tip (typically 1-nl in volume). The protein solution is then printed onto the Z-GRIP™ substrates in repeats of five for each substrate (Repetition of the protein spots is one element of quality control and will be further discussed later).

The goal of a successful protein print run is to deliver uniform and consistent volume (and, thus, mass) aliquots of protein sample to the substrate. The successful delivery of proteins is dependent upon physical characteristics of the printhead, pins, and the substrate. Proteins are heterogeneous and prone to precipitation that can clog microarray print pins (all of which were developed for nucleotides, a more homogeneous type of polymer). Therefore, the Stealth pin must be quality controlled, where quality control involves specific washing regimes and inspection under a microscope, as detailed by the manufacturer. Under a microscope, damaged pins will typically be observed to have bent or uneven contact regions, where contaminated pins will typically be seen with obstructions that clog. Proteins used are preferably dissolved in PBS, and filtered down to 0.45-μm, so that the probability of clogging in the 75-μm bore diameter of the Stealth pin is reduced. The preferred method of quality control, however, is to observe glass substrates printed with PBS. The first and last slide in the print rack or multiwell template preferably shows that every sample intended to be printed has been printed, there is no carry over, and the first and last slides have the same number and volume of spots. The pins were washed between protein runs, because proteins can precipitate and otherwise clog the Stealth channel.

Proteins in solution are a good growth source for contaminating microbes. And, the fact that recombinant proteins have been derived from bacterial sources further increases the likelihood of sample-protein contamination. It is, therefore, desirable to maintain as much of a sterile environment within the robotic microarray printer as possible. Equipment is thoroughly cleaned with 70% ethanol after all print runs, especially those involving potentially contaminated protein samples. Additionally, printing proteins at temperature of between about 4° C to 8°C was found to reduce microbial growth and maintain protein integrity, as proteins denature, are prone to proteolysis, etc., at higher temperatures (specifically at 25° C to 37° C). Printing at 4 to 8°C also helped retain the primary, secondary, and tertiary structure of recombinant proteins. Protein-protein interactions are dependent upon the molecule's three-dimensional shape. The application of recombinant

proteins can interfere with proper protein folding, secondary modifications, and activity, creating a challenge for protein microarrays. However, proteins printed from the same 384-wells plate at room temperature and at 8° C demonstrated the superiority of printing at the lower temperature. Proteins printed at room temperature lost all activity after two print runs (about 16-hrs), and the proteins printed at 8° C lost reactivity typically after eight print runs (about 64-hrs).

Replicate print runs took from several minutes to several hours. It is suggested that one uses calibration markers and tandem blanks throughout the microarray. Calibration markers aid in the addressing of positive signals ensures that each array print was successful, and allows for user (and other) error that may affect spot intensity to be corrected. The tandem blanks ensure that each microarray had consistent printing without carryover. Typically calibration markers were present on each array in repeats of five, throughout the array, in a pattern that allowed addressing. Calibration markers were followed by PBS blanks to ensure that there is no sample carryover. Previous work using serial dilutions of some detector molecule has found that anti-human IgG or IgE conjugated alkaline phosphatase (AP) at a dilution of 1:100 worked as a calibration marker.

Processing

We have coined the phrase "micro-multi-analyte immunoassay" (MMI) to make clear that the chemistries performed on the Z-GRIP™ in accordance with preferred embodiments of the integrated system are very similar to traditional enzyme-linked immunoassays. The principle differences are that sample mass is typically in the ng to fg range for MMI, and in the μg range for ELISA type assays (recall that protein samples are expensive and in short supply). This miniaturization increases sensitivity, and (along with the enzymatic signal) allows amplification for the assay to be conducted with crude lysate. Results indicated that colorimetric protein microarrays performed on Z-GRIP™ are approximately 50 to 1,000 times more sensitive then traditional colorimetric (alkaline phosphatase, hereafter denoted as AP) ELISA. Further details on the processing reactions are disclosed in co-pending US Pat. Appln Nos. 10/376,351, 10/422,872, and 10/431,686 and

Microarray processing for colorimetric detection takes from 1 to 3 hrs. Initially, the microarrays are blocked in any standard blocking solution, e.g., non-fat milk, BSA, or "blotto" type blockers. Blocking is one determinant to the signal-to-noise ratio, and can

proceed from 20 to 60-min. A number of different ELISA type "sandwiches" can be used to assay the recombinant library.

The microarray has applications for finding protein-protein interactions, drug-protein interactions, and autoantibody assays. For these cases, the microarray is printed onto the substrate, as previously described. The detection of these types of interactions can be accomplished in one of two ways - labeling the bait protein, or having a specific antibody directed against the bait protein and a secondary antibody containing the colorimetric enzyme (AP). Previous work has focused on finding autoantibodies (IgG or IgE) that may serve as diagnostic markers or clues to pathology.

Imaging and Analysis

In one embodiment, the present invention provides inexpensive methods for resolving colorimetric density representative of interactions between immobilized biological samples and various test substances. As used herein, biological samples refers to biological material associated with a biological material holding structure in a manner that allows for detection of the biological material, or portions thereof . Such biological material includes, but is not limited to: proteins, nucleic acids, and tissues. Biological material holding structures include, but are not limited to: microarray substrates such as a protein or DNA chip substrate or a gel. Detectable substances include, but are not limited to markers such as dyes, tags, labels, or stains. Biological samples can be detected through various means including, but not limited to the use of imaging such as direct mapping.

One or more embodiments of the present invention are operable for use in multiple imaging applications or in any other application wherein imaging of a biological sample is beneficial.. For example, such applications include, but are not limited to: imaging of two-dimensional and three-dimensional objects, such as fluorescence imaging, reflective imaging, bar code imaging, and densitometry, gel documentation. One or more of the systems and methods as described herein may be used for ultra-sensitive sample detection. One or more of the imaging systems and methods of the present invention are flexible light imaging systems with the ability to produce high-quality images. For example, various biological sample configurations can be used, including, but not limited to: single color fluorescence, multiple color fluorescence, chemi-luminescence, chemi-fluorescence, colorimetric detection, densitometry, or any other technique detectable through imaging.

Such image quality, may depend, at least in part, on the lens and electronic light detector used in such systems. Such imaging provides the ability for filmless detection.

Portions of the following description are primarily provided, for simplicity, with reference to use of microarrays such as protein chips. However, one skilled in the art will recognize that the present invention is applicable to any imageable biological sample. For example, imageable biological samples include, but are not limited to: DNA chips, 1-D gels, 2-D gels, blots, substrates having biological material thereon. In one preferred embodiment, polypeptides separated according to the independent parameters of isoelectric point and molecular weight can be imaged using the present invention.

An imaging system according to the present invention may be used to replace expensive optical detection systems currently employed for microarray analysis. In general, one embodiment of such a system may include an electronic light detector array, a filter, and, optionally, a mapping lens apparatus that enables a microarray to be mapped onto the electronic light detector array. For example, each position on the microarray surface has a corresponding position or set of positions, on the electronic light detector array. Light associated with the biological material at an address on the microarray surface is received or sensed at one or more known addressed detector pixels or set of detector pixels. Such detector systems are disclosed in U.S. Pat. Appl. No. US 2002/0018199 A1, which is hereby incorporated in its entirety by reference thereto.

In a preferred embodiment of the present invention, a reacted microarray may be developed and analyzed. For example, the microarray may be developed using a variety of applicable detection chemistries, including, but not limited to labeled antibodies and enzyme-linked assays. The microarray preferably can be analyzed by scanning the microarray using a linear array of detectors, such as, for example, a digital scanner. Such imaging equipment is inexpensive and readily available. Such scanning eliminates the need for a complicated microscope that requires maintenance and trained personnel. By eliminating many lenses, the disadvantages stemming from use of many lenses are reduced.

Acquisition

The protein microarray is preferably incubated with a sample (e.g., human serum, proteins, antibodies, drugs and other ligands) expected to interact with the immobilized polypeptides. The array is then preferably washed and then incubated with a secondary detector molecule. The detector molecule in this example is conjugated with Alkaline

Phosphatase (PA). The array is then incubated with an enzyme substrate, such as BCIP/NBT substrate. BCIP/NBT (blue-violet) is one of the most sensitive enzymatic substrates because of the significant increase in reaction product with longer incubation time. Another advantage of the BCIP/NBT substrate is that it can be dehydrated and cleared from the array after processing.

The array is then preferably washed and the precipitation reaction stopped as a result of washing away the required reagents. The array may then be air dried in a dust free environment. The array is then transported to a flatbed scanner. For example, the following scanners have been used with the following protocol and resolutions: Epson perfection 2400 and 1650, CanoScan N1240U and Hewlett Packard ScanJet 5300C. Any scanner can be used in accordance with the preferred embodiments of the present invention to capture images of the dried microarrays. Image produced using a Epson 2400 is shown in **Figure 10**.

In accordance with one mode of the present invention, preprinted labels with barcodes of specific numerical sequences are included on the microarray slides or multiwell templates. The barcodes may be read by a handheld scanner, or by the imaging software to expedite the data processing by relating each microarray with the types of protein, antibodies, patient information and the treatments stored in a database. Based on particular type of arrays, the barcodes can be divided into five or less segments corresponding to the different information. Barcodes can be used as an ID for the specific microarray. They may be etched on the slide or multiwell template, or printed on an adhesive label and applied to the slide or template. In addition, a duplicate barcode ID from a patient sample, may be transferred to the slide or multiwell template to identify the patient sample. The barcodes may also serve as a landmark for the scanning equipment and software to facilitate addressing of individual spots on the array.

The scanner mode is preferably set to a high resolution, preferably about 1600 dpi. The choice of the scanning resolutions depend on the needs. Lower resolutions offer faster scanning, smaller image file sizes, but lower image qualities. Workable settings are 600dpi, 800dpi, 1200dpi, 1600dpi and 2400dpi. It is preferred to observe the whole scanning area by using the previewing mode prior to scanning. Using conventional zoom settings, the system can be used to select and zoom into specific areas of interest containing the desired microarray spots. Once the areas of interest are visable in the previewing mode,

the microarray can be scanned and the images can be saved on a directory for subsequent visualization and analysis. Preferably, the imaging station includes a computer which is programmed to automate the scanning protocols.

Another option is to use a commercial software program, e.g., SPOTWARE Software (Telechem, Sunnyvale, CA; a software package designed specifically to acquire microarray images) in conjunction with the flatbed scanner. This software allows for direct capturing of the microarray, without the hassle of previewing the whole scanner area and then zooming in to scan the whole chip. Instead, it is possible to preview just the chip, and to zoom into a particular area of the chip. Settings include a choice of '16-bite grayscale' or '24-bite color', and 'invert light to dark' or 'view as false color.' Typical settings use 24-bite color viewed as false color at 1600-dpi (where dpi is set on the scanner). The false color distinguishes positive signals very clearly, making it easier on the eye and to analyze. Once the slide or multiwell template is previewed, specific portions of the microarrays can be viewed and saved. The SPOTWARE program gives a signal to noise ratio of 16,000 to 1, and a resolution of 10-μm. From here, images can be saved on a directory for subsequent visualization and analysis.

Analysis

In one preferred embodiment of the present invention, image analysis software is used to analyze the microarray data. Scanned images are preferably opened and the average intensity of each spot is determined with the background contributions eliminated. The steps of analysis include, but are not limted to: addressing or gridding the spots, segmentation to distinguish the foreground from the background, and intensity extraction and data storage. Suitable software has been developed for image analysis. There are a number of software packages that can accomplish this, including Adobe PHOTOSHOP (6.0 or higher), ARRAYVISION, and IMAGETOOL.

When opening the scanned images in PHOTOSHOP, typically the first step is to adjust the autolevels of the microarray chip. Then, depending upon whether or not the image was acquired via the flatbed scanner, the color may need to be inverted, to give a black background and light spots. This step is not necessary when using SPOTWARE, as images can be given in false color. If desired, the image may be zoomed into, to get a clearer image of the spots, and to aid in the next step. Then, using the rectangular marquee

tool, individual spots are highlighted, and the histogram observed. The mean value of the luminosity is then recorded.

The marquee can then be dragged over the next positive spot, and the luminosity for this, recorded. For PHOTOSHOP, the same marquee is preferably just dragged over the spot of interest, thereby keeping the amount of pixels being observed consistent. The marquee is preferably also dragged over the background so that spot values can be normalized against this. Typically, the background value is close to, if not equal to, 0. Once the luminosity of the series of spots has been recorded (each protein is preferably spotted in replicates, e.g., 2-10 times), the average value is taken, and the background, subtracted. This gives a single intensity value for each spotted protein.

For ARRAYVISION, the steps of analyses include addressing or gridding the spots, segmentation to distinguish the foreground from the background, as well as the intensity extraction and data storage. Examples of gridding to assist in spot data analysis is illustrated in **Figure 11**. Suitable software are developed for the image analyses.

The extracted intensity of the spots are analyzed by querying the database. The spots related to the targets are selected and their intensities may be compared with the threshold values. When the intensities are found to be above the thresholds, the software raises a flag or a warning to inform the user about a possible positive sample. It is also possible to first invert the colors in PHOTOSHOP and then open the image in ARRAYVISION, or to use the false color image scanned via SPOTWARE.

IMAGETOOL has many advantages over the other two analysis software packages. Once in the program, the user simply needs to open the image, select the analyze points option, and click on points within the microarray chip. The program will automatically record both the location of the selected point on the chip, along with three values of the intensity within the selected point.

Another advantage of this program is that, in conjunction with the flatbed scanner, it can acquire the image directly from the scanner. IMAGETOOL will go directly to the scanner program so that the image can be scanned as normal. Once scanned, the image automatically opens in IMAGETOOL to be analyzed.

In another preferred embodiment, the extracted intensity of the spots are analyzed by querying the database. The spots related to the targets are preferably selected and their intensities may be compared with the threshold values. When the intensities are found to

be above the thresholds, the software preferably provides a warning to inform the user about a possible positive sample.

Regardless of which software is used, a first step to quantification in accordance with a preferred embodiment of the disclosed method is to input all lumens values into a spread sheet, such as Microsoft EXCEL, and if necessary, average these values to one number per spot. In general, quantification occurs by first determining the average intensity value for each protein, along with its standard deviation can be determined. These intensity values can be converted into mass values, thus quantifying protein hybridization. Automation of these steps can be accomplished by programming in accordance with the disclosed operations by those skilled in the art.

More specifically, each analyzed microarray has a quantification series, where the quantification series is the known mass of the measured protein. Typically, the series uses known proteins ranging from mass 0-pg to 25-pg. As the amount of measured protein increases, so does the lumen value.

For example, the IgE antibody binds in a 1:1 ratio with the OVA allergen. Then, a calibration curve is first created for IgE by plotting the average intensity as a function of the known mass.

Once a calibration curve has been created, the IgE binding to OVA can be quantified. After analyzing the data for a dilution of OVA (ranging from a 1:10,000 to 1:1,000 titer), the lumens values are converted into mass values. These values are obtained by utilizing a calibration curve, as it gives a relation between the signal intensities and protein mass. Then, the mass of IgE bound to OVA as a function of dilution can be plotted.

Our colorimetric assay differs from traditional fluorescent microarrays, in that it does not require a fluorometer but still has good signal-to-noise properties. One of the advantages of the colorimetric system is the ability to use a low cost scanner with modified gain and custom software. This scanning system has been found to be sensitive, quantitative, and have low background. It also makes it reasonable for someone to order custom printed arrays, or print there own on a low cost printer, and do the detection for several thousand dollars (vs. 10 to 50 times this cost for a fluorescent or CCD imaging system).

While a number of preferred embodiments of the invention and variations thereof have been described in detail, other modifications and methods of using the disclosed system and components thereof will be apparent to those of skill in the art. Accordingly, it should be understood that various configurations, modifications, and substitutions may be made of equivalents without departing from the spirit of the invention or the scope of the claims. Further, it should be understood that the invention is not limited to the embodiments set forth herein for purposes of exemplification, but is to be defined only by a fair reading of the appended claims, including the full range of equivalency to which each element thereof is entitled.

All of the references cited herein are incorporated in their entirety by reference thereto.

WHAT IS CLAIMED IS:

1. A system for printing, processing and imaging biomolecular interactions, comprising:

a template comprising at least one well configured to hold a microarray substrate;

a printing station comprising an automated microarray printer in a climate-controlled compartment for applying a plurality of biomolecular markers in an addressable manner to said microarray substrate;

a processing station comprising an automated liquid dispensing and mixing robot for adding, mixing and/or extracting liquid reagents from said microarray substrate, wherein said liquid reagents comprise at least one test sample and a developing reagent, said developing reagent being capable of generating a detectable signal when said at least one test sample binds to one of the plurality of biomolecular markers on said microarray substrate; and

an imaging station comprising a flatbed digital scanner for digitizing the detectable signal(s) such that a digitized image of said microarray substrate is formed, and a microprocessor comprising imaging software adapted to quantify and analyze the digitized image.

2. The system of Claim 1, further comprising a mechanized conveyor system adapted to transport said template from said printing station to said processing station to said imaging station.

3. The system of Claim 2, further comprising control software with a user interface, said control software being adapted to provide the user with hands-free control of printing, processing, imaging and transport parameters.

4. The system of Claim 1, wherein the climate-controlled compartment regulates at least one of temperature, humidity, and gas content.

5. The system of Claim 4, wherein the climate-controlled compartment maintains the temperature between about 0° C and about 37° C.

6. The system of Claim 4, wherein the climate-controlled compartment maintains the temperature between about 4° C and about 8° C.

7. The system of Claim 1, wherein the automated microarray printer utilizes a printing buffer to apply the plurality of biomolecular markers to said microarray substrate.

8. The system of Claim 7, wherein said printing buffer comprises KPO_4, NaCl, methanol, and DTT.

9. The system of Claim 1, wherein said microarray substrate is mounted on a column within said at least one well.

10. The system of Claim 1, wherein said microarray substrate further comprises a PVDF membrane.

11. The system of Claim 1, wherein said at least one well further comprises a drain configured to facilitate removal of liquid reagents from said microarray substrate during processing.

12. The system of Claim 1, wherein said processing station further comprises a vacuum source adapted to facilitate vacuum-assisted drainage and vacuum drying of said microarray substrate.

13. The system of Claim 1, wherein said processing station is further adapted to position a protective cover over said template.

14. The system of Claim 13, wherein said protective cover is adapted to seal said at least one well.

15. The system of Claim 13, wherein said protective cover further comprises sealable ports adapted to allow a tubular member from said automated liquid dispensing and mixing robot to enter said at least one well in order to add, mix and/or extract liquid reagents from said microarray substrate.

16. The system of Claim 1, wherein said imaging station further comprises a cooling device.

17. The system of Claim 1, wherein said biomolecular markers are proteins or nucleic acids.

18. The system of Claim 1, wherein said biomolecular markers are antibodies, antigens or allergens.

19. The system of Claim 1, wherein said template comprises from 1-96 wells.

20. The system of Claim 1, wherein said developing reagent comprises an enzyme-linked antibody adapted to generate a colorometric change upon addition of a substrate.

21. A system for processing and imaging biomolecular interactions, comprising:

a template comprising at least one well configured to hold a pre-printed microarray substrate, said microarray substrate comprising a plurality of biomolecular markers immobilized in an addressable manner on a PVDF membrane;

a processing station comprising an automated liquid dispensing and mixing robot for adding, mixing and/or extracting liquid reagents from said microarray substrate, wherein said liquid reagents comprise at least one test sample and a developing reagent, said developing reagent being capable of generating a detectable signal when said at least one test sample binds to one of the plurality of biomolecular markers on said microarray substrate; and

an imaging station comprising a flatbed digital scanner for digitizing the detectable signal(s) such that a digitized image of said microarray substrate is formed, and a microprocessor comprising imaging software adapted to quantify and analyze the digitized image.

22. The system of Claim 21, further comprising a mechanized conveyor system adapted to transport said template from said processing station to said imaging station.

23. The system of Claim 22, further comprising control software with a user interface, said control software being adapted to provide the user with hands-free control of processing, imaging and transport parameters.

FIG. 1

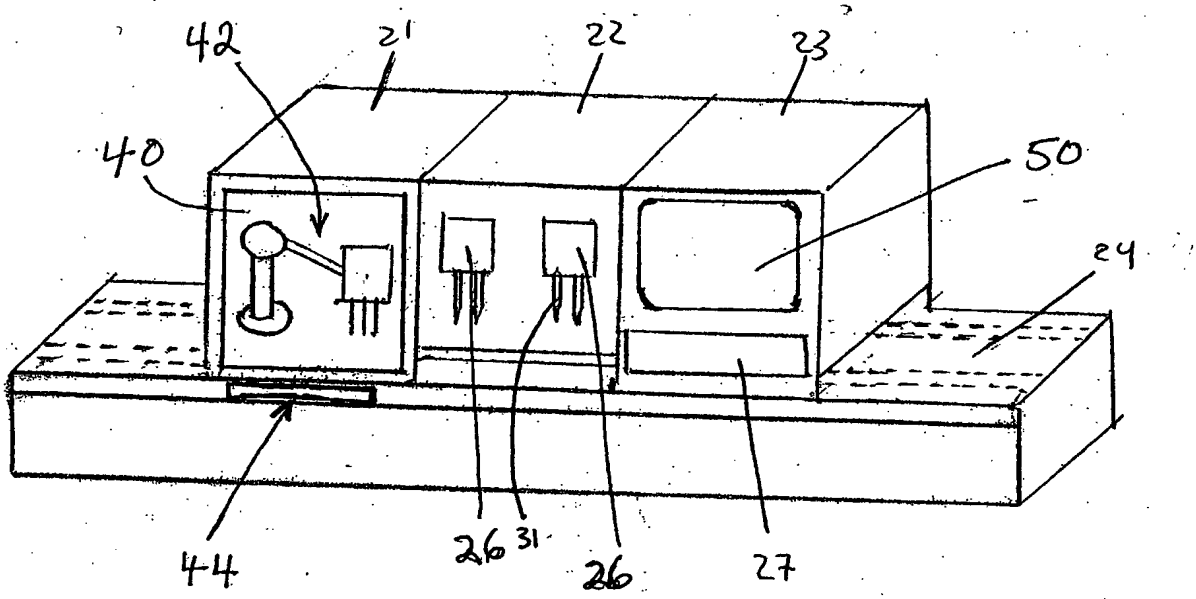
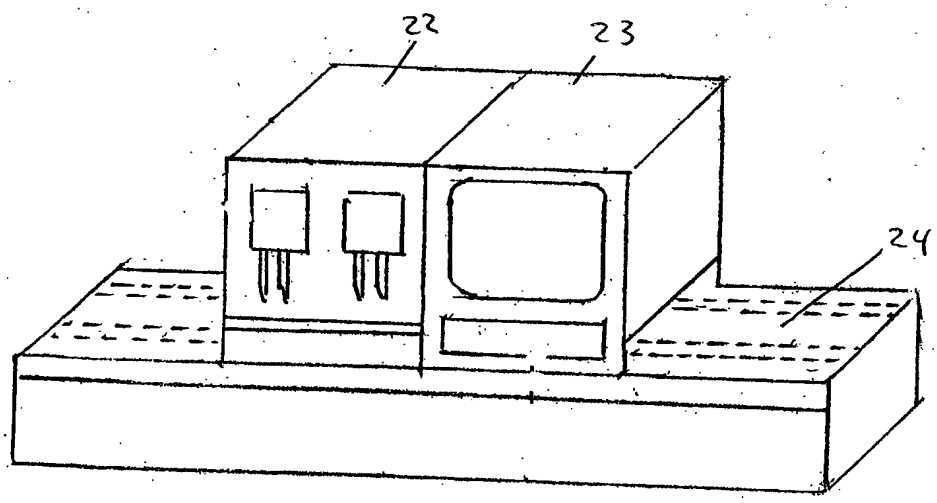


FIG. 2



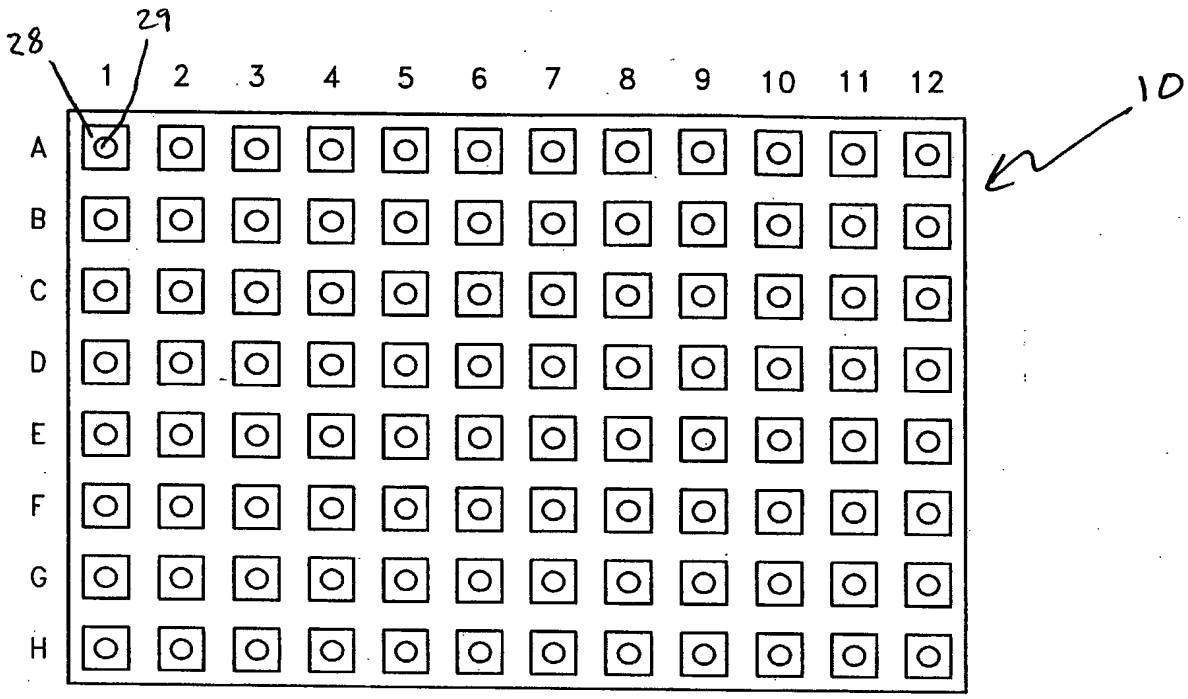


FIG. 3

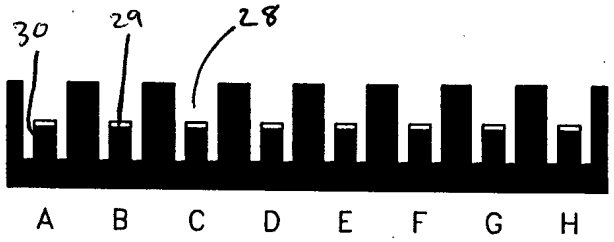


FIG. 4

FIG. 5

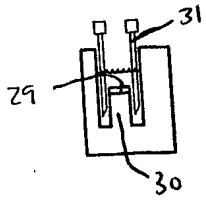


FIG. 6

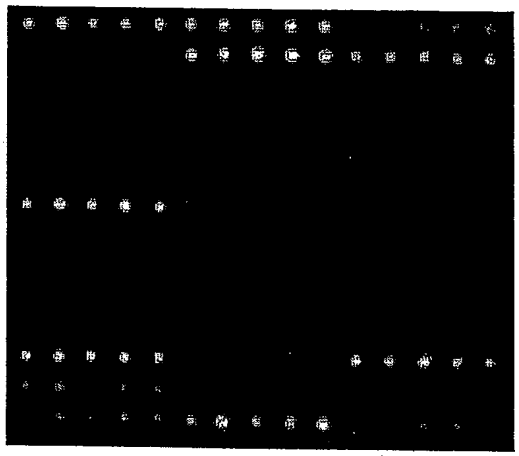


FIG. 7

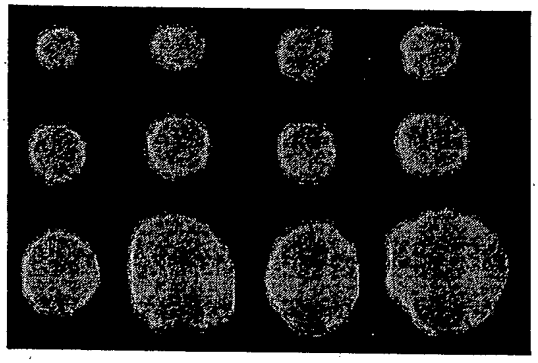


FIG. 8

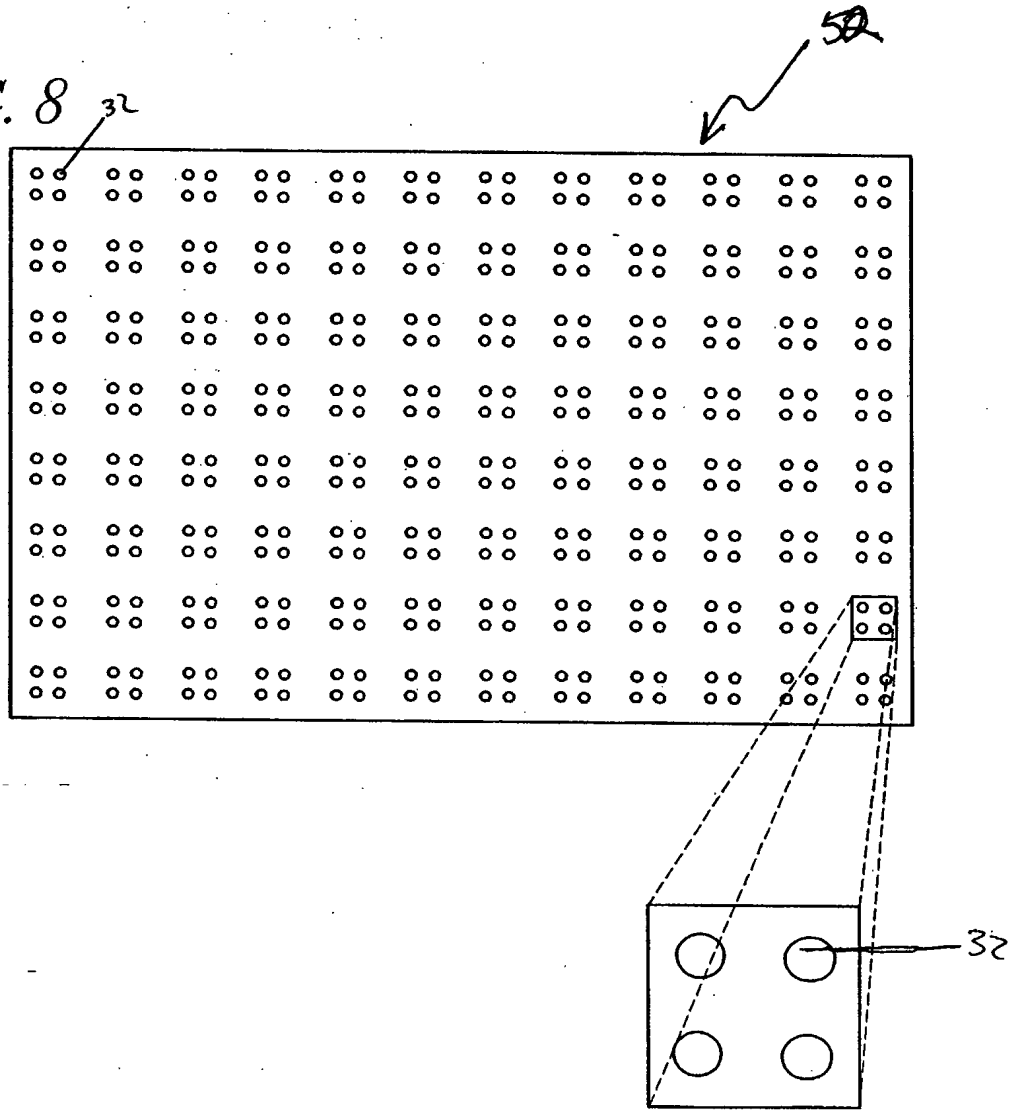


FIG. 9

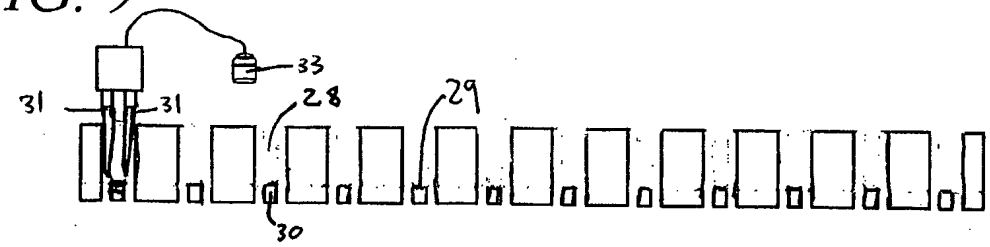


FIG. 10

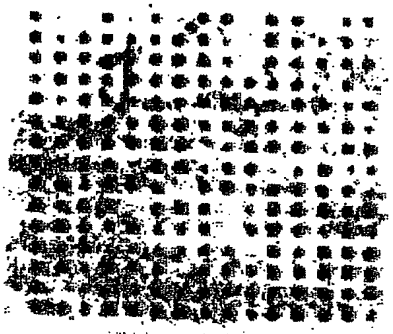
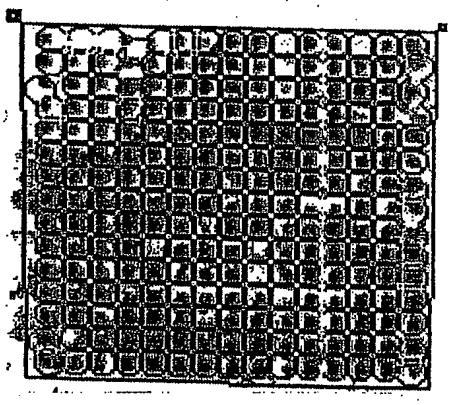


FIG. 11



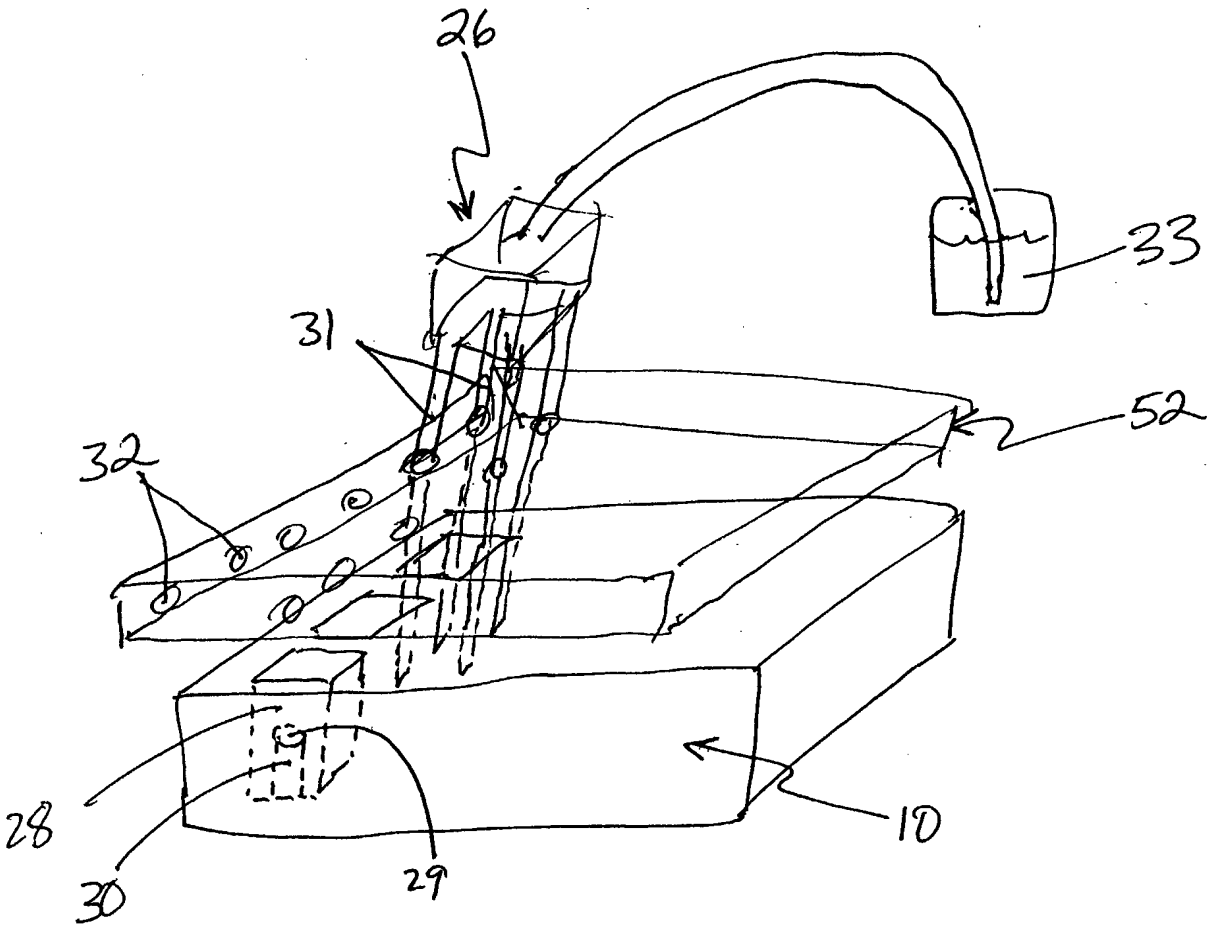


Figure 12